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Bovine serum amine oxidase and spm potentiate docetaxel and interferon- α effects in inducing apoptosis on human cancer cells through the generation of oxidative stress

M. Marra^a, A. Lombardi^a, E. Agostinelli^b, G. Giuberti^a, S. Zappavigna^a, G. Tempera^b, G. Vitale^c, M. Bifulco^d, A. Abbruzzese^a, M. Caraglia^{a,*}

^a Department of Biochemistry and Biophysics, School of Medicine, Second University of Naples, Via Costantinopoli 16, 80138 Naples, Italy

^b Department of Biochemical Sciences "A. Rossi Fanelli" and National Research Council (CNR), Biology and Molecular Pathology Institutes, University of Rome "La Sapienza", Rome, Italy

^c Section of Endocrinology, University of Milan, San Luca Hospital, Italian Auxologic Institute, Milan, Italy

^d Department of Pharmaceutical Sciences, University of Salerno, Fisciano, Italy

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ABSTRACT

It was previously demonstrated that bovine serum amine-oxidase (BSAO) and SPM (SPM) addition to cancer cells induces cell growth inhibition and over-run the multi-drug resistance (MDR) phenotype through the oxidative stress caused by polyamine metabolites. In this study, it is reported that BSAO/SPM enzymatic system antagonizes the survival pathway induced by either docetaxel (DTX) or interferon alpha (IFN α) in human epidermoid cancer KB cells. The combination of BSAO/SPM with either DTX or IFN α had a synergistic effect on cell growth inhibition through apoptosis in both human epidermoid KB and breast cancer MCF-7 cell lines. The effects of the BSAO/SPM-DTX combination on apoptosis were caspase 3 and 9-dependent and were paralleled by the enhancement of intracellular O $_2^{2-}$, nitric oxide levels and of lipo-oxidation. The scavenger moiety *N*-acetyl-cysteine antagonized the effects on apoptosis and cell growth inhibition induced by the combination suggesting a role of the oxidative products of SPM. These effects occurred together with a decrease of the physiological scavenger MnSOD and an increase of both p38 kinase activity and DNA damage. The results suggest that DTX and IFN α could sensitize tumour cells to the oxidative stress and apoptosis induced by BSAO/SPM through the induction of a survival ras-dependent pathway and the consequent elevation of the intracellular polyamine pool. These data allow the design of new therapeutic strategy based on the use of this combination in human neoplasms.

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1. Introduction

Polyamines can induce apoptosis, necrosis, inhibition of cell proliferation and inhibition of DNA and protein synthesis through the generation of intracellular oxidative stress. A way to achieve these effects is the addition of bovine serum amine oxidase (BSAO), a copper-enzyme (EC 1.4.3.6.), to polyamines. BSAO is a glycoprotein of 170 kDa MW, which oxidatively deaminates the primary amino groups of SPM and spermidine. The reaction involves oxygen and water as substrates and leads to the formation of H $_2$ O $_2$, aldehydes and ammonia. In the case of SPM, the monoaldehyde, the unstable dialdehyde or a further breakdown product, likely to be acrolein, are formed. In the enzymatic reaction catalyzed by BSAO only H $_2$ O $_2$ and aldehyde(s) are responsible for cell killing demonstrating a potent anti-tumour activity. Moreover, SPM and BSAO addition to tumour cells is able to antagonize the multi-drug resistant phenotype [1]. In

fact, the enzymatic SPM metabolites, H $_2$ O $_2$ and aldehyde, sensitized both sensitive and multidrug resistant cancer cells to the exposure to chemotherapy agents [2–4].

In this regard, drug combinations in cancer therapy could be useful to obtain improved clinical responses as well as the ability to use lower concentrations of the drugs thus reducing their side effects. On the other hand, anti-cancer agents able to induce oxidative stress are available. In this view, both docetaxel (DTX) and interferon alpha (IFN α) increase the level of intracellular peroxy radicals and lipid peroxidation products [5]. Based on these data, the combination of BSAO/SPM enzymatic system with different anti-cancer drugs as IFN α or DTX could be synergistic in terms of cytotoxicity and apoptosis of human cancer cells.

DTX is an antineoplastic agent belonging to the taxoid family that promotes the assembly of tubulin into stable microtubules essential for mitotic and interphase cellular functions, while simultaneously inhibiting their disassembly in the absence of GTP [6]. IFN α is a biological agent widely used in the therapy of several neoplasms [7–11]. IFN α is an important regulator of cancer cell growth and differentiation, affecting cellular communication and

* Corresponding author. Tel.: +00390815665871/4; fax: +00390815665863.

E-mail addresses: michele.caraglia@unina2.it, michele.caraglia@alice.it (M. Caraglia).

signal transduction pathways and produced during the immunological control of tumor growth [11]. The way by which tumor cell growth is suppressed by IFN α is not well known. However, it is reported that IFN α induces apoptosis in human squamous cancer [12,13], glioma [14] and virus-infected cells [15]. Therefore, it is likely that this cytokine acts, at least in part, through the triggering of programmed cell death [16]. In previous *in vitro* studies carried out on human epithelial tumour cells, both DTX and IFN α induced caspase 3-dependent apoptosis and an increased ras and Erk activity suggesting the simultaneous onset of a homeostatic anti-apoptotic pathway in either DTX or IFN α -treated cancer cells [17]. The specific disruption of this pathway with agents raised against ras (dominant negatives or farnesyltransferase inhibitors) enhanced apoptosis and anti-tumour activity of both DTX and IFN α [16–18].

Based on these results, in the present paper, we have investigated if the cytotoxicity induced in human head and neck KB and breast MCF-7 cancer cells by DTX or IFN α could be enhanced by BSAO/SPM enzymatic system and if this combination could antagonize the ras-dependent survival pathway in these cells. This study was performed in order to both detect a new mechanism of cancer cell growth regulation and to design a new anti-tumour intervention strategy.

2. Material and methods

2.1. Materials

DMEM, BSA and FBS were purchased from Flow Laboratories (Milan, Italy). Tissue culture plasticware was from Becton Dickinson (Lincoln Park, NJ). Interferon alpha-2b recombinant was a gift of Schering (Schering-Plough, New Jersey, USA). Rabbit antisera raised against α -tubulin, pERK-1 K-23, ERK1/2 C-14 and monoclonal antibodies (Mab) raised against anti-p38 and anti-phospho p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-3,-8,-9, anti-Akt Mab and the relative activity evaluation kit, were purchased by Cell Signalling (Cell Signaling Technology, MA, USA). Anti-pan-Ras clone 10 Mab and the relative activity evaluation kit were purchased from Calbiochem (Darmstadt, Germany). Anti-HSP-70 C92F3A-5 and anti-HSP-90 AC88 Mab were from StressGen Biotech. Co. (Victoria, BC, Canada) and anti-HSP-27 G3.1 Mab was from Affinity Bioreagents (Neshanic Station, NJ). Rabbit antisera raised against PARP, pH2AX (Ser139) and MnSOD antibodies were from Upstate Biotechnology (Upstate, Milan, Italy). The thiobarbituric acid (TBA) and malondialdehyde (MDA) were purchased from Sigma AG (Sigma-Aldrich srl, Milano, Italy).

2.2. Cell culture

The human oropharyngeal epidermoid carcinoma KB and breast MCF-7 cancer cell lines, obtained from the American Type Tissue Culture Collection, Rockville, MD, were grown in DMEM supplemented with heat inactivated 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, 1% L-glutamine and 1% sodium pyruvate. The cells were grown in a humidified atmosphere of 95% air/5% CO $_2$ at 37 °C.

2.3. Purification of BSAO

Bovine blood was withdrawn at a slaughterhouse and mixed with 3.8% sodium citrate solution (an anticoagulant) and then treated according to Turini et al. [19] to purify the enzyme amine oxidase. Some modifications were made to the method: a) CM-Cellulose column, equilibrated with phosphate buffer (0.01 M) at pH 5.8, to remove haemoglobin, followed by b) an AE-Agarose column, in phosphate buffer (0.01 M) at pH 7.2 to eliminate ceruloplasmin. Finally, two ionic exchange chromatographies were performed using a Q-Sepharose column, in phosphate buffer (0.025 M) at pH 6.8 and a Q-

Sepharose column, in phosphate buffer (0.02 M) at pH 8.0, according to Janes et al. [20]. The enzyme was eluted highly purified with a NaCl gradient. All purification steps were carried out in a cold room, at 4 °C.

2.4. Drug combination studies

For the study of the synergism on growth inhibition, KB and MCF7 cells were seeded in 96-multiwell plates at the density of 5×10^3 cells/well. After 24 h incubation at 37 °C the cells were treated with different concentrations of BSAO, SPM, DTX and IFN α . Drug combination studies were based on concentration-effect curves generated as a plot of the fraction of unaffected (surviving) cells versus drug concentration after 48 h of treatment. Assessment of synergy was performed quantitating the combination index (CI) derived from the median-effect principle of Chou and Talalay using the CalcuSyn software (Biosoft, Ferguson, MO) [21]. The isobologram method is a graph representation of the pharmacologic interaction and is composed by the CI/effect curves that represent the CI versus the fraction of cells affected/killed by the drugs in combination. Combination data points that fall on the line (CI=1) represent an additive drug-drug interaction, whereas data points that fall below (CI<1) or above the line (CI>1) represent synergism or antagonism, respectively. The dose reduction index (DRI) represents the measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level compared with the doses of each drug alone. Furthermore, we analyzed the specific contribution of both DTX and IFN α on the cytotoxic effect of the combination of drugs by calculating the potentiation factor (PF), defined as the ratio of the IC $_{50}$ of either DTX or IFN α alone to the IC $_{50}$ of DTX or IFN α in combination with SB mixture as described before; a higher PF indicates a greater cytotoxicity [22].

2.5. Western blot analysis

KB and MCF7 cells were grown for 48 h with or without IFN α or DTX and/or SPM/BSAO solution at 37 °C. For cell extract preparation, the cells were washed twice with ice-cold PBS, scraped and centrifuged for 30 min at 4 °C in 1 ml of lysis buffer (1% Triton, 0.5% sodium deoxycholate, 0.1 mM NaCl, 1 mM EDTA, pH 7.5, 10 mM Na $_2$ HPO $_4$, pH 7.4, 10 mM PMSF, 25 mM benzamidin, 1 mM leupeptin, 0.025 U/ml aprotinin). Equal amounts of cell proteins were separated by SDS-PAGE, electrottransferred to nitrocellulose and reacted with the different antibodies. Blots were then developed using enhanced chemiluminescence detection reagents (SuperSignal West Pico, Pierce) and exposed to X-ray film. All films were scanned by using Quantity One software (BioRad laboratories, Hercules, CA).

2.6. Measurement of polyamine content

KB and MCF-7 cells were treated with DTX or IFN α for 48 h and then harvested by scraping. Polyamines were quantified using reverse-phase HPLC with fluorimetric detection after derivatization with o-phthalaldehyde as previously described [23].

2.7. Flow cytometric analysis of apoptosis

Annexin V-FITC (fluorescein isothiocyanate) was used in conjunction with a vital dye, Propidium Iodide (PI), to distinguish apoptotic (Annexin V-FITC positive, PI negative) from necrotic (AnnexinV-FITC positive, PI positive) cells. Briefly, cells were incubated with AnnexinV-FITC (MedSystems Diagnostics, Vienna, Austria) and propidium iodide (Sigma, St. Louis, MO, USA) in a binding buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl $_2$, 2.5 mM CaCl $_2$) for 10 min at room temperature, washed and resuspended in the same buffer. Analysis of apoptotic cells was performed by flow cytometry (FACScan, Becton Dickinson). For each sample, 2×10^4 events were acquired.

Analysis was carried out by triplicate determination on at least three separate experiments.

2.8. AKT kinase assay

KB cells were treated with BSAO-SPM solution and/or DTX as described above. At the time of processing 1 ml ice-cold Cell Lysis Buffer (20 mM TRIS, pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β glycerophosphate, 1 mM sodium orthovanadate, 1 μ g/ml leupeptine, 1 mM PMSF) was added to cells that were incubated on ice for 10 min. The cells were collected and transferred to microcentrifuge tubes and centrifuged at 1200 g for 10 min at 4 °C. The supernatants were collected and precipitated with 20 μ l of IgG1 anti-Akt monoclonal antibody immobilized with agarose beads (Cell Signaling Technology, MA, USA) by o/n incubation with gentle rocking at 4 °C. The resulting immunoprecipitates were then incubated for 30 min at 30 °C with 1 μ g GSK-3 fusion protein (Cell Signaling Technology) in the presence of 200 μ M ATP and Kinase Buffer (25 mM TRIS, pH 7.5, 5 mM β -glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$). The reaction was terminated with the addition of 20 μ l 3 \times SDS sample buffer. The supernatants were boiled for 5 min and electrophoresed by 12% SDS-PAGE and the protein electro-transferred on a nitrocellulose film. Phosphorylation of GSK-3 was detected using as probe an anti-Phospho-GSK-3 α/β (Ser21/9) rabbit polyclonal antibody and then with a secondary anti-rabbit HRP-conjugated monoclonal antibody. The film was washed with TBS 1 \times –0.05% Tween 20 buffer and the specific reactivity was detected by chemiluminescence technique (Amersham) according to the manufacturers instructions (Cell Signaling Technology, MA, USA).

2.9. Lipid peroxidation measurement

The binding of thiobarbituric acid to malondialdehyde-bis-(dimethylacetal) 1,1,3,3-tetramethoxypropan (MDA) formed during lipid peroxidation results in a chromogenic complex. The level of lipid peroxide under treatment, was determined by measuring thiobarbituric acid reactive substances (TBARS) according to the method of Esterbauer and Cheeseman (20diKI). Briefly, the homogenate was obtained by lysing 1–5 10^6 cells with 10% ice-cold TCA. The lysis mixture was centrifuged at 800 g for 10 min. Aliquots (1 mL) of supernatant were added to 1 ml of 0.6% 2-thiobarbituric acid (TBA) and heated in a boiling water bath for 10 min. Basal levels of TBARS were measured fluorimetrically (excitation at 530 nm, emission at 550 nm) while TBARS levels induced by different drugs were measured spectrophotometrically at 532 nm. MDA was used as standard. TBARS levels were normalized for cell protein content.

2.10. Spectrophotometric determination of NO

The determination of serum NO concentration, expressed in μ M, has been performed with an indirect method through the measure of serum concentrations of nitrates and nitrites. For NO determination the spectrophotometric reaction of Griess was used [24].

2.11. Determination of caspase activity

Cells were seeded and treated as described before. At the time of caspase determination, cells were lysed. For each reaction, 50 μ l of cell lysate was added to 1 ml reaction mixture composed of reaction buffer, DTT 10 mM and the specific substrate peptide DEVD for caspase-3 conjugated to 7-amino-4-trifluoromethylcoumarin (AFC). The mixtures were incubated for 1 h at 37 °C. The levels of free AFC were measured using a spectrofluorometer with an excitation wavelength of 400 nm and an emission wavelength range of 480–520 nm (peak at 505 nm).

2.12. Statistical analysis

All data are expressed as mean \pm SD. Statistical analysis was performed by analysis of variance (ANOVA) with Neumann–Keul's multiple comparison test or Kolmogorov–Smirnov where appropriate.

3. Results

3.1. SPM/BSAO combination with either Docetaxel or IFN α antagonizes activation of the Ras-dependent survival pathway in human epidermoid KB cancer cells

We have recently demonstrated that both IFN α and DTX induce an EGF-Ras \rightarrow Erk-dependent anti-apoptotic signalling in human epidermoid cancer cells (17). Based upon these results, we have investigated if the SPM/BSAO mixture could have synergistic effects on cell growth inhibition in combination with either DTX or IFN α . We have found that the single agents and the SPM/BSAO mixture did not affect the expression of Ras (Fig. 1A). However, DTX and IFN α used alone for 48 h induced an about 1.5–2-fold increase of the Ras activation ratio (calculated as the ratio between the intensities of the bands associated with ras activity and expression, respectively). SPM/BSAO mixture induced only a slight increase of ras activity. On the other hand, SPM/BSAO mixture completely antagonized the effect of

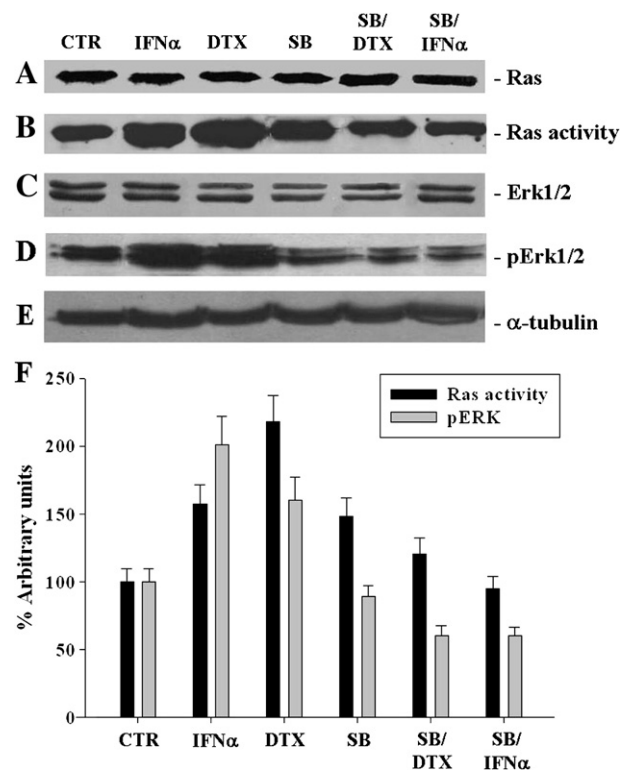


Fig. 1. Effects of SPM/BSAO mixture and Docetaxel or IFN α on Ras-dependent pathway. (A) Western blot assay for the expression of the total ras protein. (B) Affinity precipitation of ras performed with the minimal binding domain of raf-1 conjugated with agarose microspheres for the evaluation of ras activity as described in "Materials and methods". Determination of the expression (C) and phosphorylation (D) of erk-1 and 2 evaluated after blotting with an anti-MAPK and an anti-pMAPK specific Mab, respectively, as described in "Materials and methods". (E) Expression of the house-keeping protein α -tubulin, used as loading control. (F) Laser scanner of the bands associated to ras activity and pErk. The intensities of the bands were expressed as % arbitrary units. Bars, SEs. The experiments were performed at least three different times and the results were always similar. CTR, untreated cells; DTX, 48 h 0.23 ng/ml Docetaxel; IFN α , 48 h 200 UI/ml Interferon-alpha; SB, 48 h 3×10^{-3} EU/ml BSAO+ 0.075 mM SPM; SB/DTX, 48 h SB+ 0.23 ng/ml Docetaxel; SB/IFN α , 48 h SB+ 200 UI/ml Interferon-alpha.

Table 1
Effect of DTX and IFN α on polyamine content in KB and MCF-7 cells

Polyamine content (nmol/mg of protein)			
Cells	Treatment	Spermidine	SPM
KB	Control	26.9 \pm 2.7	9.2 \pm 2.5
	DTX	39.7 \pm 3.1	17.2 \pm 2.3
	IFN α	41.7 \pm 3.8	19.4 \pm 2.8
MCF7	Control	22.3 \pm 1.5	9.8 \pm 0.8
	DTX	41.8 \pm 2.1	18.1 \pm 1.7
	IFN α	43.5 \pm 1.4	19.9 \pm 2.6

DTX, docetaxel; IFN α , interferon alpha. The differences between treated and untreated cells were all significant ($p < 0.02$).

DTX and IFN α , thus reducing Ras activity to the levels of untreated cells (Fig. 1B and F).

Thereafter, we have evaluated the effects of two drugs alone or in combination with SPM/BSAO mixture on the terminal enzymes of the survival MAPK pathway, Erk-1 and Erk-2. We have found that the drugs added alone induced a 1.5–2-fold increase of Erk-1/2 activity as evaluated with a western blotting assay using an antibody raised against the phosphorylated/activated isoforms of the two enzymes (Fig. 1D and F). The combination of the agents caused an about 50% reduction of the activity of the two enzymes (Fig. 1D and F). On the other hand, at the same experimental conditions neither the single agents nor the combination induced substantial changes of Erk-1/2 expression (Fig. 1C), thus suggesting a direct effect on enzyme activation induced by the upstream regulators rather than on enzyme expression/content.

3.2. Effect of DTX and IFN α treatment on KB and MCF-7 cell polyamine pools

After proving that DTX and IFN α induced an increased activity of Ras and Erk1/2 in human epidermoid KB cancer cells, we next evaluated whether the level of polyamine pool could be also changed by the pharmacological treatment in these cells. Human epidermoid oropharyngeal (KB) and breast cancer (MCF-7) cell lines treated with DTX or IFN α showed an increase of polyamine content (Table 1). In detail, both DTX and IFN α induced a 1.5 and 2-fold increase in spermidine content in KB and MCF-7 cells, respectively, and an about 2-fold increases in the SPM content (Table 1). An about 45% increase of both SPM and spermidine contents was found in both cell lines exposed to the combination of DTX and SPM/BSAO mixture while an about 15% increase was evident in cells treated with the mixture alone (data not shown). Similar results were also obtained on putrescine content (data not shown).

3.3. The SPM/BSAO mixture synergize with either DTX or IFN α on KB and MCF-7 cell growth inhibition

On the basis of the previous results, we have studied the effect of different concentrations of SPM/BSAO mixture (SB) in combination with either IFN α or DTX on proliferation of human epidermoid carcinoma cell lines, KB and MCF-7. We have performed these experiments with MTT assay and the resulting data were elaborated with the dedicated software Calcsyn (by Chou and Talalay, see also "Materials and methods"). In details, to investigate the relative contribution of each agent to the synergism, serial dilutions from different mixtures of the two drugs (SB plus IFN α or DTX) were tested using equipotent doses of the two agents (50:50 cytotoxic ratio) or

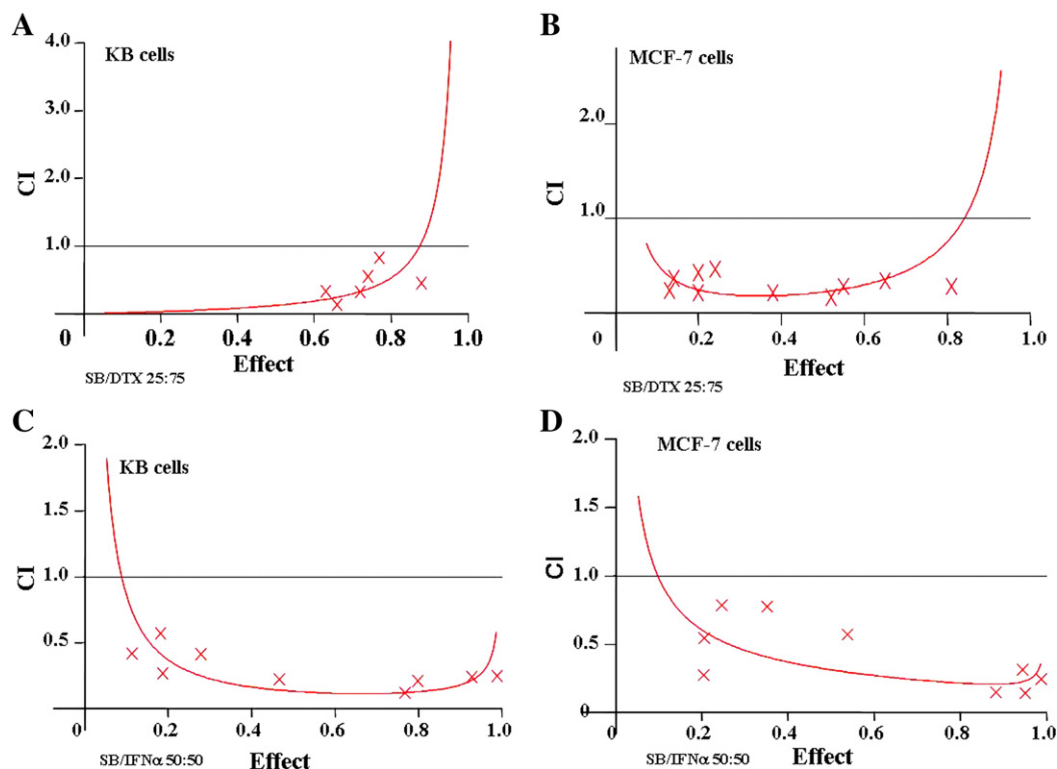


Fig. 2. SPM/BSAO mixture and Docetaxel or IFN α have a synergistic effect on human epidermoid cancer cell growth inhibition. We have evaluated the synergistic antiproliferative effect induced by Docetaxel or IFN α in combination with SPM/BSAO mixture (SB) at 48 h on KB and MCF7 cells. CI/fractional effect curves show the CI versus the fraction of cells affected/killed by Docetaxel or IFN α in combination with SPM/BSAO mixture (SB) for the different cell lines. Two molar ratios were used for both cell lines: higher relative doses of DTX (IC₂₅ of SB/IC₇₅ of DTX) (A and B) and equitoxic concentrations of SB and IFN α (at the ratio of their IC₅₀s) (C and D) on KB (A and C) and MCF7 cells (B and D), respectively. CI, combination index. Each point is the mean of at least four different replicate experiments. Combinations were highly synergistic when CIs were < 0.5 .

higher relative doses of DTX (75:25 cytotoxic ratio). Typical examples of CI/fractional effect curves showing the CIs versus the fraction of cells affected/killed by IFN α or DTX in combination with SB are illustrated in Fig. 2 for the different cell lines. The CI values, computed at the 50:50 and 75:25 cytotoxic ratios and calculated at concentrations resulting in 50% of cell kill (CI₅₀) for both SB/DTX and SB/IFN α are reported in Table 2.

We have found that the combination of SB/DTX was highly synergistic when the drugs were used at higher concentrations of DTX on both KB and MCF-7 cell lines (Fig. 2A and B, respectively) with CI₅₀s (the combination index calculated for 50% cell survival by isobologram analysis) of 0.126 and 0.25 for KB and MCF-7 cells, respectively (Table 2). On the other hand, synergistic effects were obtained at equitoxic concentrations of IFN α and SB on both cell lines (Fig. 2C and D) with CI₅₀ of 0.132 for KB cells and of 0.314 for MCF-7 cells (Table 2). In our experimental conditions, the DRI₅₀ of DTX were equal to 9.08 in KB and 2.5 in MCF-7 cells, respectively, when DTX was used at ratios with higher concentrations (Table 2). Moreover, the DRI₅₀ of IFN α was equal to 16.06 in KB cells and of 27.05 in MCF-7 cells, when the drugs were used at equitoxic ratios (Table 2). Moreover, values of potentiation factor (PF) reported in Table 2 demonstrated that IFN α had an important contribution to the cytotoxic effect of the combination in KB cells. These results demonstrate that a very strong synergism can be recorded on cell proliferation when anti-cancer drugs are used in combination with the SPM/BSAO enzymatic system. Effective concentrations of both DTX and IFN α in the combinatory experiments can be reached in vivo.

3.4. Combination between SPM/BSAO mixture and DTX increases apoptosis in epidermoid cancer cells

We have selected the combination of SPM/BSAO mixture and DTX that were synergistic at Calcsyn elaboration and we have evaluated the apoptotic effects of their combination on KB cells through FACS analysis (Fig. 3A–D) and fluorescence microscopy (Fig. 3G) after double labelling with annexin V and Propidium Iodide (PI).

We have found that the treatment with SPM/BSAO mixture and DTX alone induced apoptosis in only 11–15% of cell population compared to untreated cells as demonstrated with FACS analysis (Fig. 3A–C, E and F). However, when the cells were treated with the drugs in combination 36% of apoptosis was found (Fig. 3D–F). Analogous results were obtained with experiments of fluorescence microscopy after PI and annexin V labelling. Double labelling showing apoptotic cells was evident after exposure to the combination of SPM/BSAO mixture and DTX (Fig. 3G). The addition of DEVD-fmk, which is a caspase-3 inhibitor, to the cells treated with the different agents completely antagonized the apoptosis suggesting caspase 3 involvement in cell death caused by the combination (Fig. 3F). These data suggest that the synergism on cell growth inhibition induced by SPM/

BSAO mixture and DTX could be mediated, at least in part, by the induction of apoptosis in human epidermoid KB cancer cells. In fact, the combination potentiated apoptosis induced by the single agents and the synergism recorded on cell growth inhibition could be due to the onset of apoptosis and other cell death mechanism that were not investigated in the present manuscript.

3.5. Effects of the different combinations on caspase activation and PARP fragmentation

In order to characterize the apoptosis induced in these epithelial cancer cell lines we have evaluated if the different combinations induced the activation of mediators of the execution phase of apoptosis. We have evaluated the activation of caspase 3 with a fluorimetric assay using the synthetic substrate, DEVD-AF. The fluorescence intensity that measures the cleavage of the substrate increased after exposure to DTX and SPM/BSAO mixture and was potentiated in cells treated with the combination (Fig. 4A). This effect was specifically antagonized by the addition of the caspase-3 inhibitor DEVD-fmk (Fig. 4A). In the same experimental conditions, we have evaluated the effects of the different combinations on the cleavage and the consequent activation of caspase 9, 3 and 8 with western blotting. Caspase 8 was determined using a specific antibody that recognizes only the intact form of the enzyme. We have found that the single treatments increased the cleavage of caspase 3 and 9 (Fig. 4B and C, respectively) and the combination enhanced this effect (Fig. 4B and C, respectively). On the other hand, the different treatments caused no changes of the levels of uncleaved caspase 8 (Fig. 4D). These effects suggest that the apoptosis induced by combination was largely due to caspase 9 activation, as upstream caspase, and to caspase 3 stimulation, as terminal caspase. Therefore, apoptosis was likely paralleled by the activation of a mitochondrial apoptotic pathway. Moreover, we have evaluated the effects of the combination on PARP cleavage that occurs in the final stages of apoptosis, as evaluated with a western blotting using an antibody that recognizes both PARP fragments. We have found that single agents caused slight PARP fragmentation that was again increased by the combination (Fig. 4E).

3.6. Effects of SPM/BSAO mixture in combination with DTX on lipid peroxidation and NO concentration

On the basis of the well known ability of SPM and BSAO to induce oxidative stress through production of toxic aldehydes and consequent intracellular ROS elevation, we have evaluated the effects of combination on both lipid peroxidation and nitric oxide production (Fig. 5A and B, respectively). We have found that single agents induced an about 2-fold increase of lipid peroxidative damage that was increased by the concomitant treatment (Fig. 5A). The addition of 20 mM *N*-acetylcysteine (NAC) completely antagonized the effect

Table 2

Combination Index (CI)^a and Dose Reduction Index (DRI)^b values according to the different cytotoxic ratios and potentiation factor (PF)^c values of DTX and IFN α

Cell lines	25:75 Cytotoxic ratio			50:50 Cytotoxic ratio		
	SB/DTX			SB/IFN α		
	CI ₅₀ (\pm SD)	DRI ₅₀ DTX (\pm SD)	PF DTX (\pm SD)	CI ₅₀ (\pm SD)	DRI ₅₀ IFN α (\pm SD)	PF IFN α (\pm SD)
KB	0.126 (0.1156)	9.082 (0.86)	4.7 (0.41)	0.132 (0.0423)	16.064 (1.25)	28.47 (0.35)
MCF-7	0.25 (0.44)	14.42 (1.33)	2 (0.156)	0.314 (0.046)	27.05 (2.752)	35.3 (4.16)

CIs less than 0.5 indicated strong synergism. The linear correlation coefficient (*r*) for the fitting between CIs and fractional effects was always between 0.96 and 0.99, indicating that the data were statistically accurate. DTX, docetaxel; SB, SPM/BSAO mixture; IFN α , interferon alpha.

The Friedman nonparametric rank test was used to analyze the impact of the different cytotoxic ratios on the whole cell line panel, *p*=0.018.

^a CI values (mean \pm standard deviation from at least three separate experiments performed in quadruplicates) represent the assessment of synergy induced by drug interaction. Combination index (CI) values of <1, 1, and >1 indicate synergy, additivity, and antagonism, respectively.

^b DRI values (mean \pm standard deviation from at least three separate experiments performed in quadruplicates) represent the order of magnitude (fold) of dose reduction obtained for IC₅₀ (DRI₅₀) in combination setting compared with each drug alone. 50:50 cytotoxic ratio, equipotent doses of the two agents [SB (IC₅₀)/IFN α (IC₅₀)]; 25:75 cytotoxic ratio, evaluation of CIs at higher relative doses of DTX [SB (IC₂₅)/DTX (IC₇₅)].

^c PF values (mean \pm standard deviation from at least three separate experiments performed in quadruplicates) defined the specific contribute of IFN α or DTX evaluated as the ratio of the IC₅₀ of IFN α or DTX to the IC₅₀ of SB mixture.

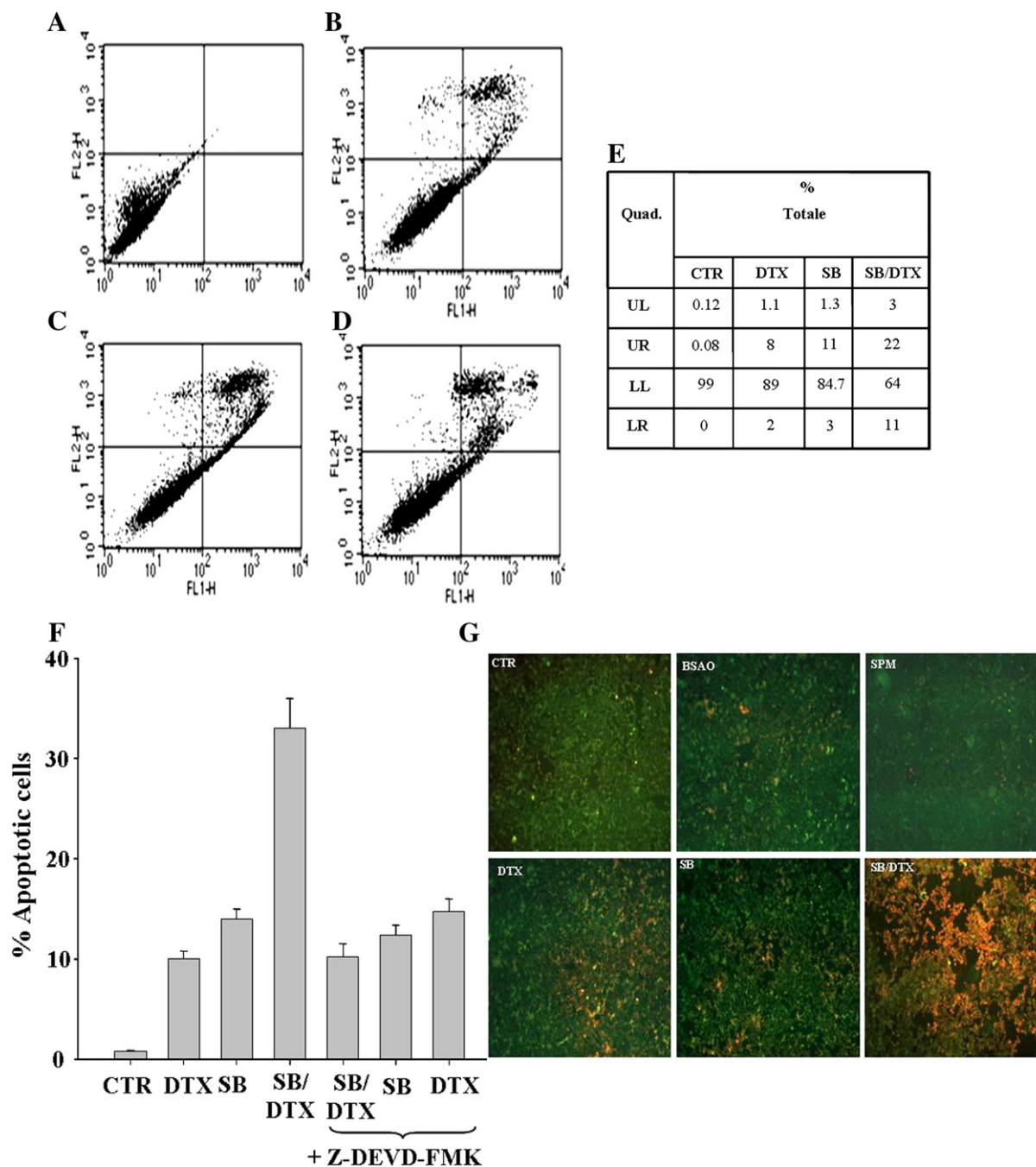


Fig. 3. Apoptotic effects of SPM/BSAO mixture and DTX combination on human epidermoid KB cells. (A–D) FACS analysis after double labelling with Annexin V and Propidium Iodide of KB cells treated with 3×10^{-3} EU/ml BSAO + 0.075 mM SPM and/or 0.23 ng/ml Docetaxel for 48 h. (A) Untreated cells; (B) 48 h 0.23 ng/ml Docetaxel; (C) 48 h 3×10^{-3} EU/ml BSAO + 0.075 mM SPM; (D) 48 h SB + 0.23 ng/ml Docetaxel. The experiments were performed at least three times and the results were always similar. (E) Percentage of cells for each quadrant. Lower left (LL) are both Annexin V-FITC and PI negative; upper left (UL) are PI positive indicating necrosis; lower right (LR) are Annexin V-FITC positive indicating early apoptosis; upper right (UR) are both Annexin V-FITC and PI positive indicating late apoptosis. CTR, Untreated cells; DTX, 48 h 0.23 ng/ml Docetaxel; SB, 48 h 3×10^{-3} EU/ml BSAO + 0.075 mM SPM; SB/DTX, 48 h SB + 0.23 ng/ml Docetaxel. (F) Percentage of apoptotic cells after double labelling with Annexin V and Propidium Iodide of KB cells treated with 3×10^{-3} EU/ml BSAO + 0.075 mM SPM and/or 0.23 ng/ml Docetaxel for 48 h and exposed to caspase-3 inhibitor Z-DEVD-FMK. CTR, Untreated cells; DTX, 48 h 0.23 ng/ml Docetaxel; SPM, 48 h 0.075 mM SPM; SB, 48 h 3×10^{-3} EU/ml BSAO + 0.075 mM SPM; SB/DTX, 48 h SB + 0.23 ng/ml Docetaxel. (G) KB cells fixed and processed at fluorescence microscopy after PI and anti-annexin V antibody labelling as described in “Materials and methods”. The experiments were performed at least three different times and the results were always similar. Double labelled cells (yellow) are apoptotic cells. Bars, SEs. The experiments were performed at least three different times and the results were always similar. CTR, Untreated cells; DTX, 48 h 0.23 ng/ml Docetaxel; SPM, 48 h 0.075 mM SPM; SB, 48 h 3×10^{-3} EU/ml BSAO + 0.075 mM SPM; SB/DTX, 48 h SB + 0.23 ng/ml Docetaxel.

induced by the combination (Fig. 5A). The single agents did not induce changes in the production of intracellular NO that was about 3-fold increased by the combination and partially antagonized by the addition of 20 mM NAC (Fig. 5B). Flow cytometric analysis of cells exposed to hydroethidine showed an increase of fluorescence intensity in cells exposed to the combination, as compared to the

fluorescence of untreated cells or cells treated with the single agents (Fig. 5C). Hydroethidine operates effectively as a probe for measurement of $O_2^{\cdot -}$ and, therefore, these data suggested that the increase of both lipid peroxidation and NO production was likely due to the increase of intracellular $O_2^{\cdot -}$ recorded in cells treated with SB and DTX. In fact, the mean fluorescence intensities (MFIs) of the cells treated

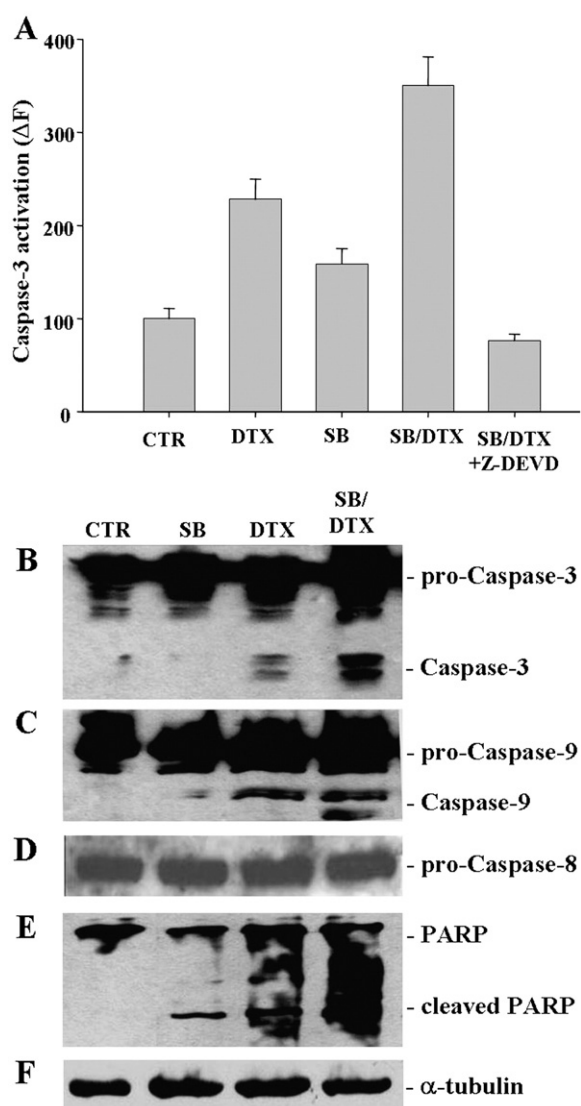


Fig. 4. Modulation of caspases 3, 8 and 9 and PARP cleavage induced by SPM/BSAO mixture and DTX combination. The cells have been cultured for 48 h with 3×10^{-3} EU/ml BSAO+ 0.075 mM SPM and/or 0.23 ng/ml DTX. The specific caspase 3 activity (A) was determined with a fluorimetric assay as described in the "Materials and methods" section. Western blot assay for the determination of (B and C) both intact and cleaved isoforms of caspase 3 and caspase 9 as evaluated after blotting with an anti-caspase-3 and an anti-caspase-9 specific Mab, respectively, as described in "Materials and methods"; (D) pro caspase-8 as evaluated after blotting with an anti-caspase-8 specific Mab that recognise only the intact form of caspase; (E) PARP fragmentation. The experiments were performed at least three different times and the results were always similar. CTR, Untreated cells; DTX, 48 h 0.23 ng/ml Docetaxel; SB, 48 h 3×10^{-3} EU/ml BSAO+ 0.075 mM SPM; SB/DTX, 48 h SB+ 0.23 ng/ml Docetaxel; SB/DTX+Z-DEVD-FMK, 48 h SB+ 0.23 ng/ml Docetaxel exposed to caspase-3 inhibitor Z-DEVD-FMK.

with BSAO/SPM or DTX alone were 66.52 ± 5.6 and 71.35 ± 6.3 , respectively, (vs. 51.4 ± 3.7 of control cells) while MFI in cells exposed to the combination was 198.6 ± 15.8 . In the same experimental conditions, the addition of NAC to cells treated with SB/DTX combination was also able to completely antagonize the apoptosis induced by the combination (Fig. 5D). Therefore, apoptosis and increase in intracellular oxidative stress were likely correlated.

3.7. Effects of SPM/BSAO mixture in combination with DTX on stress-activated signal transduction pathways

On the bases of these data, we have studied the effects of the different treatments on molecules involved in the regulation of stress-activated signal transduction pathways. We have firstly evaluated the

effects of the different treatments on the expression of HSP70 and 90 in KB cells by western blot assay (Fig. 6A and B, respectively). We have found that exposure of cells to SPM/BSAO mixture and DTX alone or in combination did not modulate the expression of these proteins (Fig. 6A and B). On the other hand, the single agents induced an about 1.5-fold increase of p38 kinase activity that was enhanced by the combination (an about 2-fold increase) suggesting a triggering of stress-dependent kinase pathways in SB/DTX-treated KB cells (Fig. 6D). These effects occurred without change in total p38 expression (Fig. 6C). Moreover, the expression levels of the scavenger protein MnSOD were almost unchanged in KB cells treated with the single agents but strongly decreased (almost undetectable) in KB cells exposed to SB/DTX combination (Fig. 6E). In conditions of high intracellular oxidation, as recorded in SB/DTX treated KB cells, was evidenced by an increase in the phosphorylated H2AX (Fig. 6F). Taken together, these data suggest that SB/DTX treatment generated an increase of intracellular ROS (directly and/or through the decrease of scavenger systems) that, in turn, activate p38 kinase or directly cause DNA damage leading to apoptosis (Fig. 7). Moreover, the increase in intracellular ROS can be also induced by oxidation of intracellular polyamines by cellular polyamine oxidase (i.e. SPM oxidase) and this latter effect cannot be excluded in our experimental model (Fig. 7).

4. Discussion

Intracellular polyamine levels are involved in the regulation of cell proliferation in different manners depending on their concentration and catabolism. In fact, being positively charged, SPD and SPM interact and stabilize DNA and RNA. Electrostatic interactions through cationic amino groups and hydrophobic interactions through methylene bridging groups are dominant. Therefore, polyamines may act as ligands at multiple sites on DNA, RNA, proteins, phospholipids and nucleotide triphosphates [25]. The inhibition of the interactions between polyamines and DNA, and/or low level of SPM have been found to increase DNA instability, augmented mutagenesis and cell death. On the other hand, high intracellular levels of SPM cause an increase of its oxidative products inducing DNA damage and cell death for either necrosis or apoptosis. Therefore, the increase of *in situ* formation of cytotoxic polyamine metabolites can be important in inducing anti-cancer effects. This could be achieved using pharmacological agents [26,27].

Moreover, SPM catabolic degradation has been also found closely related to DNA oxidation, apoptosis, oxidative stress and consequent deleterious cellular effects [1,28,29]. Among these, severe changes of the mitochondrial structure, such as dilatation of the *cristae*, disruption of membranes and apoptosis occurrence were observed in both wild type and multidrug-resistant colon adenocarcinoma and melanoma cells treated with BSAO/SPM [30,31]. Interestingly, the multi-drug resistant colon cancer LoVo cells were more sensitive to cytotoxic SPM derivatives than normal counterpart and the treatment of these cells with SPM/BSAO antagonized their MDR phenotype. These effects were attributed to an earlier and higher mitochondrial membrane depolarisation, and a higher basal production of ROS [32]. The role of oxidative stress in cytotoxicity induced by SPM is additionally demonstrated by the radio-sensitization of a neuroblastoma cell line induced by the ectopic expression of murine SPM mono-oxidase (mSMO) [33]. In fact, transfected cells have increased intracellular ROS levels that sensitize tumour cells to the DNA damage induced by ionizing agents. The involvement of oxidative products of polyamines in the radiosensitization of tumor cells is supported by SPM oxidase hyperactivity in neuroblastoma cells [33]. The polyamine-dependent intracellular ROS elevation enhances also the anti-tumour activity of some cytotoxic drugs as demonstrated by the chemosensitization of MDR cancer cells [1,34]. Moreover, a synergistic pharmacologic interaction was reported between DTX and a polyamine derivative, N1,N11-DiethylnorSPM (DENSPM), that is a SPM analogue and prototype anti-cancer drug that

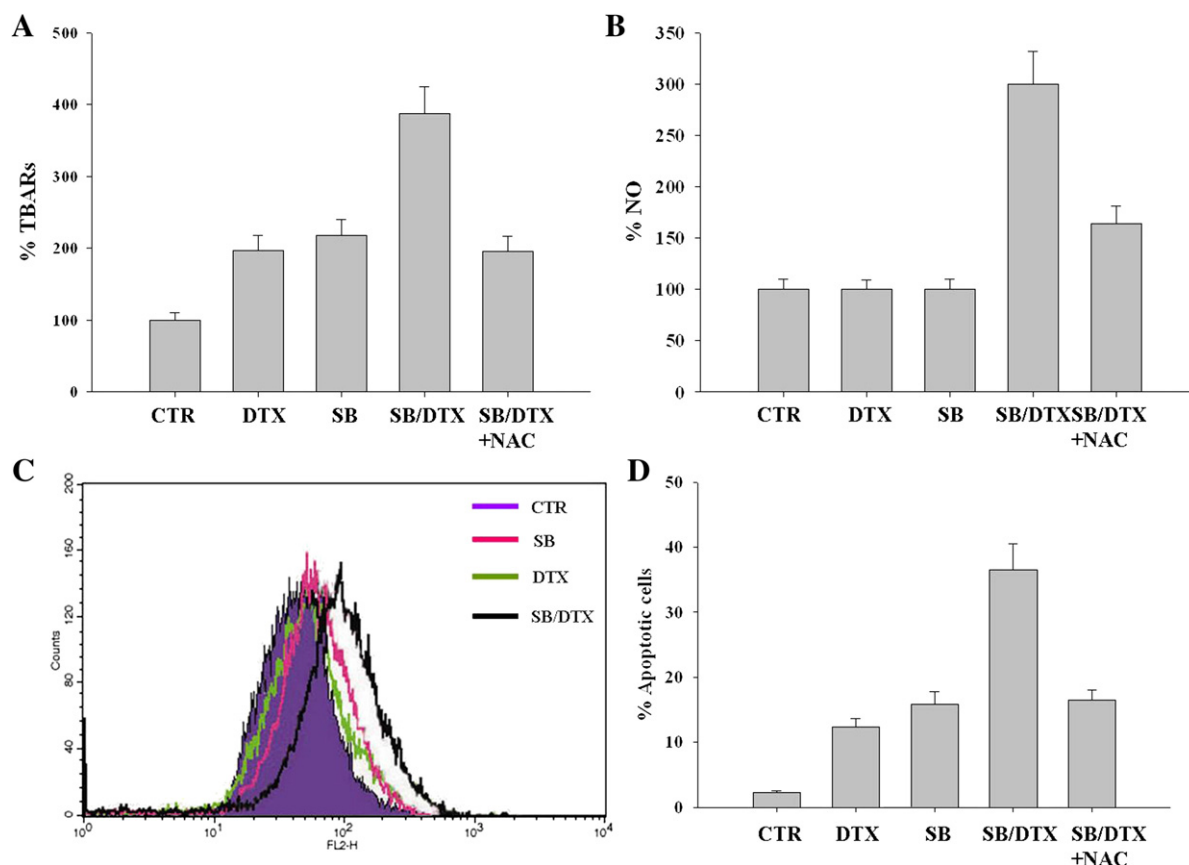


Fig. 5. Oxidative stress induced by SPM/BSAO mixture in combination with Docetaxel. Variations of (A) MDA levels expressed as % of thiobarbituric acid reactive substances (TBARS) and of (B) nitric oxide levels in KB cells after 48 h of incubation. (C) Flow cytometric analysis of KB cells exposed to hydroethidine used as a probe for measurement of O_2^- . (D) Percentage of apoptotic cells after double labelling with Annexin V and Propidium Iodide of KB cells treated with SPM/BSAO mixture and/or Docetaxel and exposed to *N*-acetylcysteine. Bars, SEs. The experiments were performed at least three different times and the results were always similar. CTR, Untreated cells; DTX, 48 h 0.23 ng/ml Docetaxel; SB, 48 h 3×10^{-3} EU/ml BSAO + 0.075 mM SPM; SB/DTX, 48 h SB + 0.23 ng/ml Docetaxel; SB/DTX+NAC, 48 h SB + 0.23 ng/ml Docetaxel + 20 mM *N*-acetylcysteine.

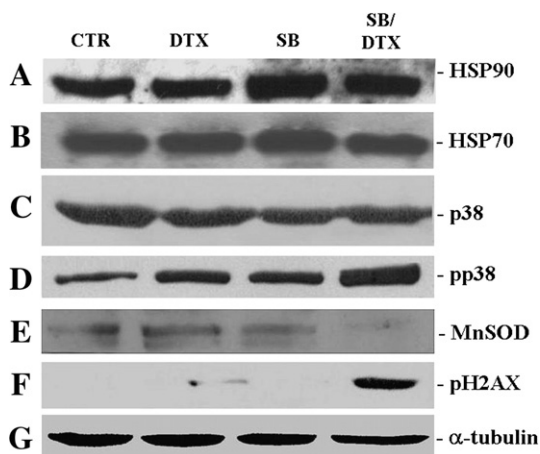


Fig. 6. Effects of SPM/BSAO mixture in combination with Docetaxel on signal transduction pathway induced by stress. Western blot assay for (A) the expression of the HSP90 and (B) HSP70 evaluated after blotting with specific antibodies as described in "Materials and methods". Determination of the expression (C) and phosphorylation (D) of p38 kinase evaluated after blotting with anti-p38 and an anti-pp38 specific antibodies, respectively, as described in "Materials and methods". Western blot assay for phosphorylated MnSOD (F) and H2AX (E) evaluated after blotting with specific antibodies as described in "Materials and methods". (G) Expression of the house-keeping protein α-tubulin, used as loading control. The experiments were performed at least three different times and the results were always similar. CTR, Untreated cells; DTX, 48 h 0.23 ng/ml Docetaxel; SB, 48 h 3×10^{-3} EU/ml BSAO + 0.075 mM SPM; SB/DTX, 48 h SB + 0.23 ng/ml Docetaxel.

depletes cellular polyamines and increases cell oxidative stress through the generation of H_2O_2 . The synergistic interaction was observed in breast cancer cell lines [35], but the mechanism of such interaction was not investigated in this report. More recently, it was reported that DENSPM reduces both expression and activity of AKT, mTOR, p70S6K and eIF-4E [36]. Moreover, the Akt-dependent pathway was recently correlated to development of resistance to DTX [37]. On the other hand, growth and survival ras-Erk and Akt-dependent pathways have a strong regulating role on polyamine metabolism. Transfection of colon cancer cells with ras oncogene enhances ornithine decarboxylase (ODC) translation that is under the control of mTOR, Akt and Erk signalling and of the phosphorylative status of eIF4E [38]. Activated Ki-ras oncogene suppresses the polyamine catabolism through the decrease of spermidine/SPM N1-acetyltransferase (SSAT) gene transcription [39]. The latter effect occurs through the down regulation of PPAR γ induced by ras-mediated activation of Erk [39]. The down-modulation of SSAT leads to an elevation of intracellular polyamine levels and above all of SPM [40].

In our experimental model cytostatic concentrations of both IFN α and DTX induced increased activity of ras and Erk, on a protective anti-apoptotic basis [16,17], and this effect was paralleled by a significant increase of intracellular polyamine levels. We hypothesize that the addition of SPM and BSAO to either DTX or IFN α -treated cells induced a synergistic effect on growth inhibition and apoptosis at least through two mechanisms: i) boosting of a strong intracellular oxidative stress that impairs the survival response occurring in tumour cells treated with cytotoxic agents or BSAO/SPM alone; ii) increase of the basal levels of polyamines mediated by the ras-dependent survival pathway caused by the cytostatic stimulus mediated by the drug. In the latter

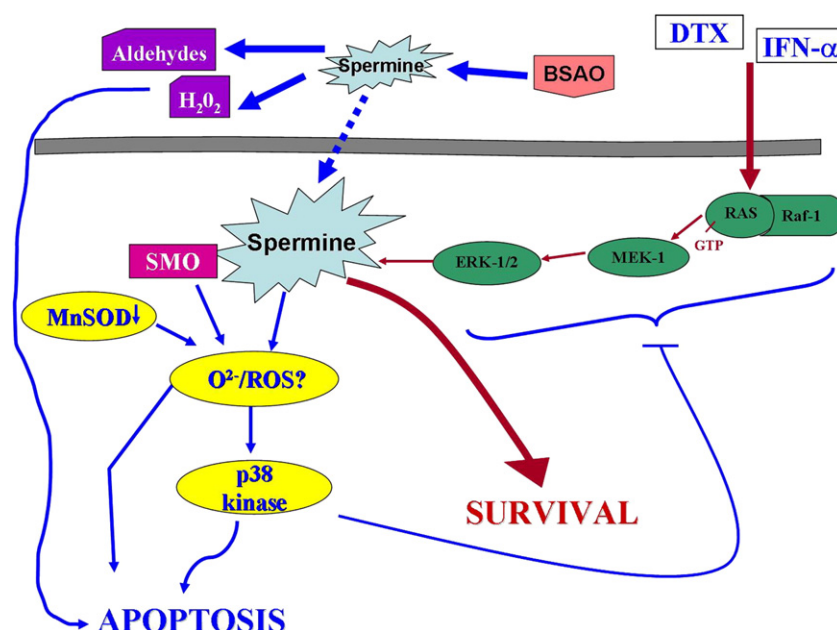


Fig. 7. The ras-Erk-dependent survival pathways induced by DTX and IFN α sensitizes tumour cells to oxidative stress induced by BSAO/SPM. DTX and IFN α cause a survival signal transduction pathway mediated by ras-MEK-1-Erk-1/2. The latter induces an increase of intracellular SPM levels (right part of the chart and red arrows). The addition of SPM/BSAO mixture induces an oxidative stress mediated by the oxidative products of SPM and this effect is enhanced by the increase of intracellular polyamine pool in either DTX or IFN α -treated cells. The formation of oxidative products is also mediated separately by the action of intracellular SPM monooxidase (SMO) or polyamine oxidase (PAO). Finally, the oxidative stress induces per se apoptosis or mediates a p38 kinase apoptotic pathway (left part of the chart and blue arrows). p38 kinase, in turn, antagonizes the ras-dependent survival pathway mediated by either DTX or IFN α through the activation of MEK-1 phosphatases.

way, tumour cells contained more substrates for intracellular polyamine oxidases (PAO and SMO) with consequent production of higher oxidative products. In turn, oxidative stress was due separately to both: i) H_2O_2 and aldehyde radicals produced extracellularly by BSAO/SPM interaction and to ii) intracellular oxidation of intracellular polyamines by PAO or SMO. Oxidative stress was paralleled by the activation of the stress-dependent p38 kinase that is also involved in the triggering of apoptotic machinery [41]. Interestingly, superoxide anions are formed from molecular O_2 by the action of intracellular oxidase. Subsequently, they are primarily degraded by conversion to H_2O_2 by superoxide dismutase [42]. H_2O_2 can be either partially converted to reactive OH^\cdot (another ROS) or in H_2O and molecular O_2 by the action of catalases. Superoxide anion inhibits MEK-1, and subsequently ERK, via a p38 kinase effect on phosphatases PP1 and/or PP2A [43]. These data support our findings regarding the overcoming of the Erk-dependent survival response occurring in IFN α or DTX-treated cells after SPM/BSAO addition.

Apoptosis induced by the combination was caspase-3 and 9-dependent and caspase-8 independent suggesting that oxidative stress triggered a mitochondrial-dependent apoptotic pathway. It was also reported that apoptosis occurring in B16-F10 melanoma cells treated with BSAO/SPM mixture was dependent on caspase-3 and -9 but not on caspase-6 [44]. Mitochondrial apoptotic pathway could be dependent upon the DNA damage induced by oxidative stress since the combination induced a strong phosphorylation of γ H2AX.

In conclusion, we describe, for the first time, that oxidative stress derived from SPM catabolism potentiated the anti-cancer activity of either DTX or IFN α . This effect was likely due to the increase of polyamine intracellular pool mediated by the hyperactivation of a survival ras-Erk-dependent signalling. These biochemical events caused strong anti-proliferative and apoptotic effects that were mediated by the activation of a mitochondrial cell death pathway possibly triggered by DNA damage induced by oxidative products. Therefore, the combination of BSAO/SPM with cytotoxic agents (such as DTX and IFN α) deserves consideration in the treatment of human oropharyngeal epidermoid and breast cancer [45]. In fact, H_2O_2 and

aldehyde(s) could be generated *in situ* by delivering BSAO into the tumor mass [46, 47] incorporating the enzyme into liposomal vesicles as previously shown with liposomal entrapment of superoxide dismutase [47]. Moreover, macromolecular anticancer drugs can be delivered also in biocompatible polymers, such as polyethylene glycol hydrogel (PEG) coated liposomes [48,49]. In this view, we are developing new BSAO delivery systems based on the encapsulation of the enzyme in tumour-specific nano-technological envelops.

Acknowledgments

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